Studies on the Relationship between the Effects of Various Substances on Absorption Spectrum of Cytochrome P-450 and the Reduction of p-Nitrobenzoate by Mouse Liver Microsomes

HENRY A. SASAME[1] AND JAMES R. GILLETTE

Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014

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SUMMARY

The reduction of *p*-nitrobenzoate by an NADPH-dependent enzyme in liver microsomes is presumably mediated by cytochrome P-450. The reaction is inhibited by substances which cause type II spectral changes in liver microsomes, but not by those which cause type I spectral changes. Type II substances inhibit only about 50% of the nitroreductase activity in liver microsomes. The apparent K_i values for the inhibition of *p*-nitrobenzoate reduction are approximately double the K_s values of the various type II substances studied. The evidence suggests that type II substances inhibit *p*-nitrobenzoate reduction by slowing the rate of cytochrome P-450 reduction.

INTRODUCTION

Liver microsomes contain NADPH-dependent enzymes that catalyze the oxidation of a variety of drugs and steroids and the reduction of azo and nitro compounds (1). These enzymatic reactions are presumably mediated by a carbon monoxide-sensitive pigment, cytochrome P-450, because carbon monoxide inhibits the oxidation of steroids (2) and most drugs (3), the reduction of *p*-nitrobenzoate (4), and a part of the reduction of Neoprontosil [disodium 2-(4'-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate] (5).

Various substrates and inhibitors of the cytochrome P-450 enzyme systems cause two types of spectral changes in liver microsomes, even in the absence of NADPH (6, 7). The type I difference spectrum is characterized by a trough at about 420 mμ and a peak at about 385 mμ, whereas the type II difference spectrum is characterized by a peak at about 430 mμ and a trough at about 390 mμ. Evidence in the present paper shows that substances which cause type II spectral changes inhibit the reduction of *p*-nitrobenzoate, whereas those which cause type I changes enhance nitro reduction. Preliminary reports of these findings have appeared (8, 9).

MATERIALS AND METHODS

Male mice (NIH general purpose, 25–30 g) were fed Purina chow diet and allowed water ad libitum. The animals were treated with daily doses of phenobarbital (80 mg/kg intraperitoneally) for 3 days, and 24 hr after the last dose were killed by breaking their necks. The livers were perfused with Krebs-Ringer-phosphate, pH 7.4, and homogenized with 4 volumes of 1.15% KCl containing 0.02 M Tris-HCl buffer, pH 7.4. Microsomes were isolated

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and nitroreductase activity was assayed as previously described (4). The incubation mixtures contained 9 mg of microsomal protein in a total volume of 3 ml. NADPH-cytochrome *c* reductase was assayed by the method of Williams and Kamin (10).

The rate of cytochrome P-450 reduction was assayed as follows. In an anaerobic cuvette (American Instrument Company, model Al-65085), 2.5 ml of liver microsomal suspension (3–4 mg of protein per milliliter) in 0.067 M sodium phosphate buffer, pH 7.4, were gassed for 7 min with oxygen-free carbon monoxide which had been passed through a solution consisting of 0.5% sodium dithionite and 0.05% sodium anthraquinone-2-sulfonate in 0.1 M sodium hydroxide. A 50-μl aliquot of 0.05 M NADPH was placed in the reagent chamber of the stopcock-plunger assembly, which was then joined to the cuvette. Oxygen-free carbon monoxide was flushed through a stopcock of the assembly for an additional 3 min. The stopcock was turned to seal the system, and the cell was placed in the cuvette holder of a Gilford spectrophotometer, model 2000, equipped with a Honeywell recorder. The NADPH solution was mixed with the microsomal suspension by depressing the plunger. The change in absorbance at 450 mμ was followed until the reaction was complete. At the end of the reaction, the stopcock assembly was removed, a few crystals of sodium dithionite were added to the experimental cuvette and an ungassed reference cuvette, and the absorbance was measured at both 450 mμ and 490 mμ for the estimation of total cytochrome P-450 content (7).

The differences between the total CO–cytochrome P-450 complex (P-450$_t$) and the amounts of CO–cytochrome P-450 measured at various times (P-450$_r$) were divided by P-450$_t$, and the resulting ratios were plotted on semilogarithmic paper. The half-time, $t_{1/2}$, for the initial phase was estimated and the rate constant, k, was calculated from the formula $k = 0.693/t_{1/2}$. Since the logarithmic plots were biphasic (Fig. 5), the k values are estimates of the initial rates of cytochrome P-450 reduction per unit of cytochrome P-450 and may not necessarily represent first-order rate constants.

Protein was assayed by the method of Sutherland *et al.* (11).

Materials. 2,4-Dichloro-6-phenylphenoxyethylamine, Lilly 390-378-23B (2,4-dichloro-6-phenylphenoxyethylmonomethylamine), and Lilly 327-169-22B (2,4-dichloro-6-phenylphenoxyethyldiethylamine) were kindly donated by Dr. Robert McMahon, Eli Lilly and Company, Indianapolis; SKF 525-A (β-diethylaminoethyl diphenylpropylacetate HCl) and SKF 26754-A (aminoethyl diphenylpropylacetate HCl) were donated by the Smith Kline and French Laboratories, Philadelphia. Desdimethylimipramine was donated by Geigy Laboratories, Ardsley, New York. All other substances were obtained commercially.

RESULTS

Type I, type II, and atypical spectral changes. As shown by Schenkman *et al.* (7), most substrates and inhibitors of the microsomal enzyme systems involving cyto-

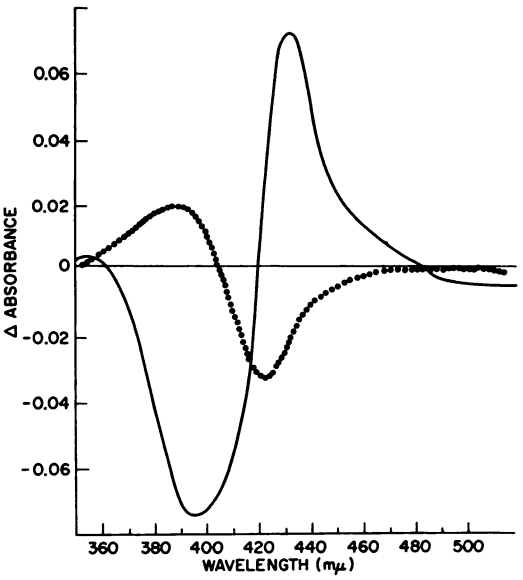

FIG. 1. *Type I and type II difference spectra*

The spectra were obtained by the procedure described in Table 1, except that the microsomal concentration was 3 mg of protein per milliliter. ····, (Lilly 327-169-22B + microsomes) − microsomes; ——, (DPEA + microsomes) − microsomes.

chrome P-450 cause either type I or type II spectral changes (Table 1 and Fig. 1). Lilly 390-378-23B, however, induces an atypical difference spectrum, characterized by a maximum at about 435 mμ and a broad minimum ranging from about 390 to 410 mμ (Fig. 2). These findings suggested the possibility that the difference spectrum caused by this compound is a composite of type I and type II spectral changes. Accordingly, on the addition of Lilly 327-169-22B, a typical type I substance, to microsomes in the reference cuvette, and of Lilly 390-378-23B to those in the experimental cuvette, the difference spectrum was typical of type II (Fig. 2). In contrast, the difference in the spectral changes caused by Lilly 390-378-23B and nicotinamide was typical of type I (Fig. 2). It seems likely that other compounds which are usually classified as type II substances may cause spectral changes which are composites of type I and type II. For this reason it is difficult to determine the relative contributions of the two types of spectral changes in producing the atypical difference spectrum. Indeed, compensation for the type II component of Lilly 390-378-23B by aniline or DPEA[2] gave smaller type I difference spectral changes than did compensation by nicotinamide.

TABLE 1

Absorption maxima and minima and relative change in absorbance of mouse liver microsomes induced by various compounds

Compound	ΔA/mg/ml	$\Delta A/\Delta A_{P\text{-}450}$	Absorption	
			Minimum	Maximum
			mμ	
Type I				
Aminopyrine[a]	0.026	0.140	420	388
Hexobarbital[a]	0.031	0.166	420	388
SKF 525-A[a]	0.028	0.150	420	388
Ethylmorphine	0.016	0.086	420	388
Lilly 327-169-22B	0.021	0.113	420	388
Type II				
Aniline[a]	0.035	0.188	395	430
Nicotinamide[a]	0.024	0.129	392	425
DPEA[a]	0.048	0.258	398	432
Pyridine[a]	0.044	0.238	393	427
Imidazole	0.059	0.316	405	433
Desdimethylimipramine	0.043	0.230	396	432
SKF 26754-A	0.031	0.166	393	430

[a] Similar results were obtained with rat liver microsomes by Schenkman *et al.* (7). The difference spectra were obtained at room temperature with a Shimadzu MPS-50L recording spectrophotometer after addition of an excess of the compounds dissolved in water to freshly prepared mouse liver microsomes (2 mg/ml) suspended in 0.067 M potassium phosphate, pH 7.4. The change in optical density (ΔA) values were determined from the absorbance of the peak or trough relative to 490 mμ. In each instance, the same volume of water was added to the reference cuvette to compensate for dilution.

Relationship between type I and type II spectral changes and effects on nitroreductase. As shown in Table 2, all the compounds that cause type II spectral changes inhibited the reduction of *p*-nitrobenzoate. Lilly 390-378-23B, which causes

[2] The abbreviations used are: DPEA, 2,4-dichloro-6-phenylphenoxyethylamine; Lilly 390-378-23B, 2,4-dichloro-6-phenylphenoxyethylmonomethylamine; Lilly 327-169-22B, 2,4-dichloro-6-phenylphenoxyethyldiethylamine; SKF 525-A, β-diethylaminoethyl diphenylpropylacetate HCl; SKF 26754-A, aminoethyl diphenylpropylacetate HCl.

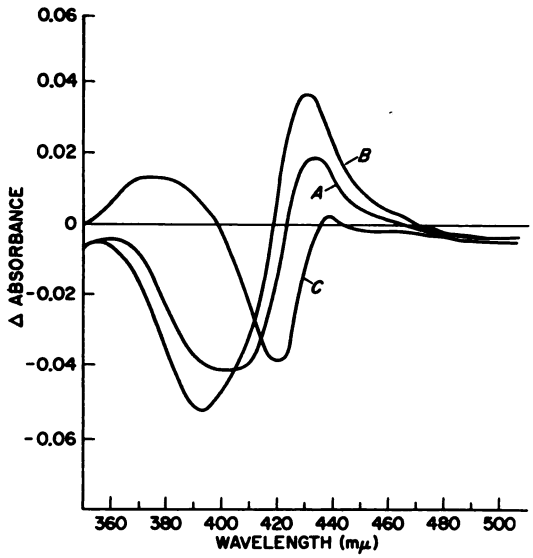

FIG. 2. *Atypical difference spectrum*

The spectra were obtained by the procedure described in Table 1 and the text. The microsomal concentration was 4 mg of protein per milliliter. Curve *A*, (50 μM Lilly 390-378-23B + microsomes) − microsomes; *B*, (50 μM Lilly 390-378-23B + microsomes) − (25 μM Lilly 327-169-22B + microsomes); *C*, (50 μM Lilly 390-378-23B + microsomes) − (3.0 mM nicotinamide + microsomes).

both type I and type II spectral changes, also inhibited nitro reduction, but to a lesser extent. In contrast, all the substances that cause only type I spectral changes enhanced rather than inhibited the reaction.

Since *p*-nitrobenzoate and *p*-aminobenzoate do not cause spectral changes in liver microsomes, and thus presumably do not displace other substances from microsomes, it seemed possible that there might be a close correlation between the apparent inhibitory constants (K_i) of the type II substances and their apparent binding constants (K_s) associated with the spectral change. Attempts to determine a value for K_i, however, revealed that DPEA exerted an unusual effect on nitroreductase. Reciprocal plots of velocity with respect to substrate concentration at various concentrations of *p*-nitrobenzoate revealed that DPEA inhibited nitro reduction by a mixed mechanism (Fig. 3), and a Dixon plot (12) of the data obtained with various concentrations of DPEA was nonlinear (Fig. 4). These findings are thus reminiscent of partial mixed inhibition (13) and suggested that the reduction of *p*-nitrobenzoate is catalyzed either by several enzymes, only

TABLE 2

Relationship between types of spectral changes caused by various compounds and their effect on nitroreductase activity in mouse liver microsomes

Compound	Concentration	Type of spectral change		Relative nitroreductase activity[a]
		Type I	Type II	
	μM			
Experiment 1				
None				100
SKF 26754-A	50	−	+	58
SKF 525-A	50	+	−	136
DPEA	50	−	+	41
Lilly 390-378-23B	50	+	+	73
Lilly 327-169-22B	50	+	−	118
Experiment 2				
None				100
Aniline	1000	−	+	80
Hexobarbital	1000	+	−	123
Ethylmorphine	1000	+	−	125
Aminopyrine	1000	+	−	105

[a] The control rates, in nanomoles of *p*-aminobenzoate formed per milligram per minute, were 3.0 for experiment 1 and 3.4 for experiment 2.

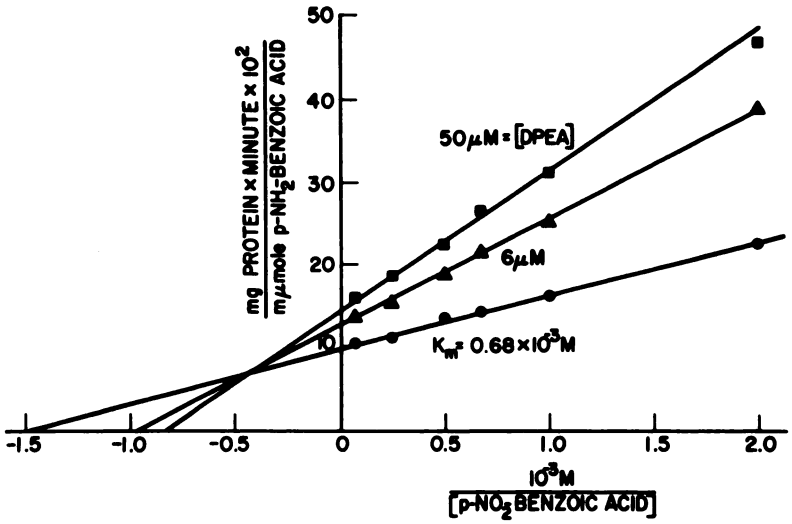

FIG. 3. *Lineweaver-Burk plot of nitroreductase activity in the presence of various concentrations of DPEA*

some of which are inhibited by DPEA, or by a single enzyme that is only partially blocked by DPEA. In neither case may the apparent K_i be easily estimated. A maximum estimate of the K_i was obtained, however, by subtracting the nitroreductase activity obtained at the highest concentration of DPEA (50 μM) from the activities obtained at other concentrations of DPEA and plotting the corrected data according to Dixon (12). As shown in Table 3, the apparent K_i was about 3.1 μM. The type II spectral changes caused by various amounts of DPEA were measured with the same protein concentration used in the nitroreductase assay. The apparent K_s value was about 2.3 μM. In similar experiments, the apparent K_i values of other type II substances were also about double their apparent K_s values. Thus, the effects of these inhibitors of nitroreductase approximate their effects on the absorption spectrum of liver microsomes.

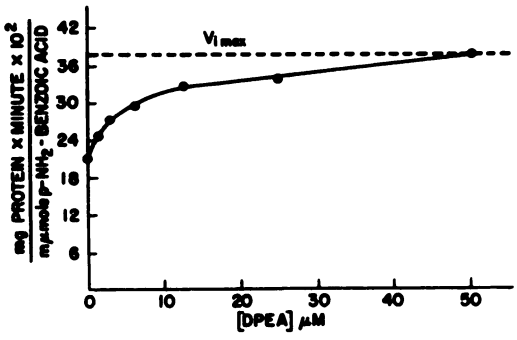

FIG. 4. *Dixon plot of nitroreductase activity in the presence of various concentrations of DPEA*

TABLE 3

Correlation of spectral constants (K_s) of various type II substances with their inhibitory constants (K_i) for nitroreductase in mouse liver microsomes

Inhibitor	K_s	K_i
	μM ± *SD*	
DPEA	2.3 ± 0.6 (3)[a]	3.1 ± 0.9 (4)
SKF 26754-A	3.5 ± 0.9 (3)	2.6 ± 0.6 (3)
Desdimethyl-imipramine	3.0 (1)	2.2 ± 0.4 (4)
Imidazole	150.0 (1)	330.0 ± 60 (3)
Pyridine	140.0 (1)	520.0 ± 20 (2)

[a] The numbers in parentheses represent the number of experiments.

Lack of effect on NADPH-cytochrome c reductase. In order to determine what part of the electron transport system was inhibited by type II substances, the effects of various type I and type II substances on NADPH-cytochrome *c* reductase were studied (Table 4). Within experimental limits, neither type I nor type II substances inhibited the enzyme.

Effect on cytochrome P-450 reduction. Since the type II substances did not inhibit

TABLE 4
Effect on rate of cytochrome c reduction in mouse liver microsomes caused by various inhibitors of drug metabolism

Inhibitor (50 μM)	Type	Cytochrome c reduced	Relative activity
		μmoles/min/mg	
None		2.75 ± 0.03	100
SKF 26754-A	II	2.63 ± 0.09	96
SKF 525-A	I	2.90 ± 0.01	105
DPEA	II	2.58 ± 0.03	94
Lilly 390-378-23B	I, II	2.35 ± 0.08	87
Lilly 327-169-22B	I	2.47 ± 0.05	90

NADPH-cytochrome *c* reduction, it seemed likely that they exerted their effects on nitro reduction by inhibiting the reduction of cytochrome P-450. As shown in Fig. 5, the reduction of cytochrome P-450 by NADPH is biphasic. Since the second phase is due mainly to the presence of trace amounts of oxygen (14), the effects of the type I and type II substances on the initial phase were studied. As shown in Table 5, DPEA and SKF 26754-A decreased the initial first-order rate constant for the reduction of cytochrome P-450 by NADPH. Moreover, DPEA also decreased the rate of cytochrome P-450 reduction by sodium dithionite. Since sodium dithionite presumably reduces the cytochrome directly, these findings suggest that DPEA exerts its effect by combining with cytochrome P-450, and not by altering an electron carrier between NADPH-cytochrome *c* reductase and cytochrome P-450.

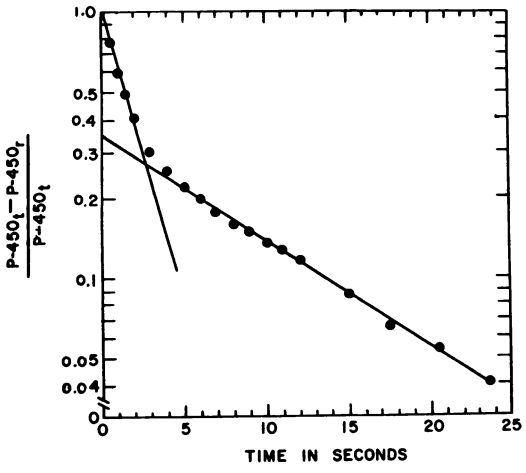

FIG. 5. *Cytochrome P-450 reduction by NADPH*
The unreduced fraction of cytochrome P-450 was calculated from the amount of CO–cytochrome P-450 formed after reduction with sodium dithionite (P-450$_t$) and the CO–cytochrome P-450 formed at various times after addition of NADPH (P-450$_r$).

TABLE 5
Effect on rate of cytochrome P-450 reduction in mouse liver microsomes caused by SKF 26754-A and DPEA and their analogues

The reduction rate of cytochrome P-450 in mouse liver microsomal suspension (9.0 mg of protein) in a final volume of 2.5 ml of 0.067 M potassium phosphate buffer, pH 7.4, in the presence of various inhibitors (50 μM) was measured as described under MATERIALS AND METHODS. The rate constant (k) was calculated from $t_{1/2}$ values obtained from the initial phase of logarithmic plots. The data represent the means of duplicate values ± half the difference between these values.

Compound	Type	k
		sec^{-1}
NADPH		0.23 ± 0.01
+DPEA	II	0.075 ± 0.01
+Lilly 390-378-23B	I, II	0.19 ± 0.01
+Lilly 327-169-22B	I	0.27 ± 0.04
NADPH		0.35 ± 0.04
+SKF 26754-A	II	0.12 ± 0.01
+SKF 525-A	I	0.27 ± 0.01
Sodium dithionite		0.30 ± 0.02
+DPEA	II	0.05 ± 0.01

In contrast, type I substances, such as ethylmorphine, Lilly 327-169-22B, and SKF 525-A, and the mixed type substance Lilly 390-378-23B did not inhibit cytochrome P-450 reduction; indeed, the type I substances may have accelerated the reduction of cytochrome P-450, though this was difficult to assess because the endogenous rate of cytochrome P-450 reduction in mouse liver microsomes approached the limit of the method.

Effects of type I and type II substances on steady-state levels of CO–cytochrome P-450. It has been suggested that oxygen inhibits the reduction of *p*-nitrobenzoic acid by competing with the substrate for reduced cytochrome P-450 (4). To determine

whether the inhibitory effects were due to reoxidation of the reduced form or to the formation of a relatively stable O_2–cytochrome P-450 complex, we tested the effects of type I and type II substances on the steady-state levels of CO–cytochrome P-450 in the presence of an NADPH-generating system and a mixture of carbon monoxide and oxygen. As shown in Table 6, type II compounds apparently decreased the steady-state level of the CO–cytochrome P-450 complex, whereas type I substances apparently increased its steady-state level. The presence of type I and type II compounds slightly altered the extinction of CO–cytochrome P-450 in a 100% CO atmosphere, suggesting that the extinction coefficients of the CO–cytochrome P-450–substrate complexes may be slightly different from that of CO–cytochrome P-450 or that trace amounts of oxygen still remain in the system. If the change in extinction were due solely to differences in the extinction coefficient, however, the ratio (B/A, Table 6) of the extinction obtained in the presence of the CO-O_2 mixture to that obtained in the presence of CO alone should be relatively constant. Since the ratios in the presence of the type II substances were lower than that of the control, it is probable that the effects of the type II substances reflect decreases in the rate of reduction of cytochrome P-450. In contrast, the ratios in the presence of type I substance were similar to those of the controls, and thus the effects of these substances on the steady-state absorbance of the CO–cytochrome P-450 complex in mouse liver microsomes may be due to differences between the extinction coefficients of the CO–cytochrome P-450 complex and the CO–cytochrome P-450–type I substance complex.

TABLE 6

Effect of inhibitor on the steady-state level of CO–cytochrome P-450 in mouse liver microsomes under a CO-O_2 atmosphere

Inhibitor (0.1 mM)	CO–cytochrome P-450[a] Air:CO = 25%:75% (B)	CO = 100% (A)	B/A × 100
	mμmoles/mg protein		
None	0.84 ± 0.01	1.26 ± 0.02	66
DPEA	0.45 ± 0.01	0.98 ± 0.05	46
Lilly 390-378-23B	0.88 ± 0.05	1.34 ± 0.05	66
Lilly 327-169-22B	0.91 ± 0.03	1.36 ± 0.04	67
SKF 26754-B	0.52 ± 0.01	1.00 ± 0.03	52
SKF-525-A	1.01 ± 0.03	1.36 ± 0.05	74

[a] An extinction coefficient of 91 mM^{-1} cm^{-1} was used to convert the extinction values to nanomoles. The data are expressed as the means of duplicate values ± half the difference between these values.

DISCUSSION

Studies on the anaerobic reduction of *p*-nitrobenzoate by liver microsomes were undertaken to clarify the mechanism of inhibition by type I and type II substances under conditions which are not complicated by the possible formation of oxidative metabolites of the inhibitors, peroxidation of lipids, or the formation of O_2–cytochrome P-450 complex. These studies revealed that nitroreductase is inhibited by substances which cause type II spectral changes, but not by those which cause type I spectral changes. The mechanism of inhibition by DPEA resembles a partial mixed type, suggesting that nitroreductase comprises a number of enzymes, only some of which are sensitive to the inhibitor, or that the inhibitor–cytochrome P-450 complex still functions as a nitroreductase but at a reduced rate. It is not possible, however, to discriminate between these mechanisms at the present time.

Since *p*-nitrobenzoate does not cause spectral changes, it seemed likely that the type II substances did not exert their action by altering the affinity of the enzyme for the substrate, but by altering the flux of electrons through the transport system. Accordingly, the rate of reduction of cytochrome P-450 by NADPH was slowed by type II substances and was either unaffected or enhanced by type I substances. Since type I and type II spectral changes presumably reflect complexes of substances with cytochrome P-450, it seemed likely that these results indicate alterations in the kinetic properties of cytochrome P-450, rather than alterations on the preceding components of the electron transport system. In accord with this view, none of the type I or type II substances used in these experiments altered the activity of NADPH-cytochrome *c* reductase in liver microsomes.

It is noteworthy that Gigon *et al.* (14, 15) have found that type II substances decrease the rate of cytochrome P-450 reduction in rat liver microsomes. In contrast to the results presented here, however, they found that all type I substances accelerate cytochrome P-450 reduction. The reason for the apparent discrepancy is unknown, but it may well be that the assay system is not sensitive enough to detect stimulatory effects of the type I substances in preparations containing high cytochrome P-450 reductase activity, or that there are species differences in the relative rates of cytochrome P-450 reduction in the presence and absence of type I substances.

The reason for the divergent effects of type I and type II substances on the rate of cytochrome P-450 reduction is unknown. Schenkman (16) has suggested, however, that type II substances form complexes with the iron in heme and hence are not reduced by NADPH. According to this view, only the unbound form of cytochrome P-450 would be reduced and hence the rate of reduction would be slowed. It is still possible, however, that the oxidized form of the cytochrome P-450–type II substance complex is reduced, but at a slower rate than the free cytochrome P-450. At the present time it is not possible to distinguish between these possibilities, but it may be important that high concentrations of type II substances do not block cytochrome P-450 reduction (15) or nitro reduction completely.

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